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Paper-based devices for rapid diagnostics and testing sewage for early warning of COVID-19 outbreak

Qingxin Hui, Yuwei Pan, Zhugen Yang *

Cranfield Water Science Institute, School of Water, Energy and Environment, Cranfield University, Cranfield, MK43 0AL, UK

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ABSTRACT

Coronavirus disease (COVID-19), caused by SARS-CoV-2, evolved into a global pandemic in 2020, and the outbreak has taken an enormous toll on individuals, families, communities and societies around the world. One practical and effective strategy is to implement rapid case identification based on a rapid testing to respond to this public health crisis. Currently, the available technologies used for rapid diagnostics include RT-PCR, RT-LAMP, ELISA and NGS. Still, due to their different limitations, they are not well suited for rapid diagnosis in a variety of locations. Paper-based devices are alternative approaches to achieve rapid diagnosis, which are cost-effective, highly selective, sensitive, portable, and easy-to-use. In addition to individual virus screening, wastewater-based epidemiology has been emerged to be an effective way for early warning of outbreak within the population, which tests viral genome sequence to reflect information on the spread and distribution of the virus because SARS-CoV-2 can be shed into wastewater through the feces and urine from infected population. In this paper, we describe paper-based device as a low-cost and rapid sensor for both diagnosis and testing of sewage for early warning of outbreak. More importantly, the device has great potential for real-time detection in the field, without any advanced facilities or well-trained and skilled personnel, and provides early warning or timely intervention of an outbreak of pandemic.

1. Introduction

The entire world has been faced with a vast pandemic, coronavirus disease 2019 (COVID-19) since 2019, and this global pandemic is still an unsolved problem and has caused a significant crisis for global public health. COVID-19 is a new respiratory infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which belongs to the family of coronaviruses. There are some other infectious diseases caused by coronaviruses in history, like severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) [1]. One defining characteristic of COVID-19 is that it spreads quickly across the world, and the outbreak with near exponential growth has put an overwhelmed strain on health systems around the world. Although countries around the world have different public health responses to this global disease because of the different stages of the outbreak, a typical effective strategy is to identify cases through rapid testing and isolate infected patients. Therefore, it is of vital importance to adopt COVID-19 testing that could accurately and rapidly detect infected individuals to reduce transmission and control outbreaks.

In a short period of time, COVID-19 has evolved into a global pandemic that has had a profoundly negative impact on individuals, families and societies around the world [2]. There have been profound changes in people's daily lives, such as telecommuting, maintaining social distance, and the inability to freely go out, and many family reunions events have been cancelled due to the ban on people gathering, like funerals and weddings. More importantly, the COVID-19 epidemic has plunged economies around the world into recession because of the adoption of lockdown measures to control virus transmission. Cultural activities in society, such as global conferences and events on fashion and sports, have been disrupted by infectious diseases. In order to alleviate the unprecedented pressures on individuals, families and societies at this difficult time, there is an urgent need for developing personal rapid diagnosis and large-scale screening tools for infections, as well as seeking effective early warning system for effective intervention and management.

2. Current state-of-art for COVID-19 diagnostics

Generally, there are mainly two categories of analytical techniques for COVID-19 detection, and the first type is the molecular approaches for

* Corresponding author.
E-mail address: zhugen.yang@cranfield.ac.uk (Z. Yang).

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the detection of viral nucleic acids. Reverse transcription polymerase chain reaction (RT-PCR) is the most widely used molecular detection technique and is regarded as the gold standard for diagnosis of SARS-CoV-2 virus [3,4]. The first step of RT-PCR assay is to collect biological fluids that may contain virus strains from the upper and lower respiratory tract, which is followed by several filtration and separation steps to isolated RNA [5]. Then, the transcriptase enzyme is used to converse viral RNA to complementary DNA (cDNA). The next step is the PCR reaction where DNA undergoes amplification with DNA polymerase. During the amplification, the TaqMan probe with a fluorescent molecule and a quencher molecule can detect specific viral sequences and generate fluorescence signal. After dozens of amplification cycles, the system can detect the intensity of the fluorescent signal, which is proportional to the virus concentration in the sample.

As a predominant diagnostic method for managing COVID-19 pandemic, RT-PCR is sensitive, specific and reliable, but it still has some unavoidable limitations. To perform the RT-PCR test, well-trained professionals, various specialists and expensive instruments are necessary, which is a concern in the developing regions and countries in short supply [6]. Also, the analysis time of RT-PCR lasts for four to six hours, and the turn-round time from sample to result usually exceeds 24 hours, which may be longer when the number of infectious cases dramatically increases [7]. More importantly, some research indicated that the diagnosis of SARS-CoV-2 by RT-PCR might be false negative at some times [8,9]. There are many possible reasons for false negative results, and the primary one is that RNA is easy to degrade, so special handling at a low temperature is needed in collection, storage, transfer, purification and processing [6].

Another molecular method that uses isothermal amplification to detect viral nucleic acids, for example, reverse transcription loop-mediated isothermal amplification (RT-LAMP). RT-LAMP depends on its ability to amplify the complementary DNA of coronavirus at a constant temperature, and the amplification needs four primers with high specificity to detect target sequences [7]. The LAMP reaction is accompanied by magnesium pyrophosphate precipitation when viral sequences are amplified, and this byproduct can increase the turbidity of the sample [3,4]. Therefore, it is possible to measure the turbidity change or employing intercalating dyes that can cause visible color change based on the increase in turbidity to monitor the amplification in real time. Since the amplification condition is at a constant temperature, this approach does not require high-cost reagents or equipment, which may reduce some financial pressures and allow testing to be carried out outside the laboratories [10]. Besides, due to the high specificity and high sensitivity, Lamb et al. suggest the use of RT-LAMP test significantly shortens the detection time to about 30 min, which is beneficial for rapid detection [11].

Apart from molecular approaches for viral nucleic acid detection, an alternative diagnose strategy is the used of immunological assays to detect various antibodies specific to SARS-CoV-2 antigens, and these assays aimed to monitor disease progression and identification of past infection [3,4]. Enzyme-linked immunosorbent assay (ELISA) is currently the most common antibody detection in use. It uses a multi-well plate coated with viral proteins, which can specifically bind antibodies from blood samples [12]. As the other antibodies labelled with enzymes are introduced subsequently, a colourimetric or fluorescent-based signal is produced.

The next-generation sequencing (NGS), also known as high-throughput sequencing (HTS), is another method for implementing COVID-19 diagnosis. This technology can determine the genome sequence of more than 1 million base pairs in a single sequencing, thus it can be used to detect the gene sequence of a virus in a short time. Previously, whole-genome sequencing method was used to identify the transmission of methicillin-resistant *Staphylococcus aureus* (MRSA) in community and hospital, and this technology showed significantly high precision and traceability in patients compared to other standard infection-control techniques [13]. In 2020, the FDA approved one product named Illumina COVIDSeq Test. This amplicon-based NGS test employs 98 DNA fragments to cover the roughly 30 kilobases in the SARS-CoV-2 genome, and the detection is usually completed within 12 hours [14]. Although the sequencing technique has high accuracy in virus diagnosis, it requires a relatively long detection time and the instruments and reagents used require high costs.

3. Wastewater-based epidemiology for early warning of COVID-19 outbreak

Although these mentioned assay techniques are evolving with improved approaches and can be well applied in COVID-19 diagnosis, it is still an enormous challenge for the globe to screen every suspicious infection in such a critical time. To address this issue, it is believed that wastewater-based epidemiology (WBE) is an alternative method for predicting virus spread and early warning pandemic by detecting pathogens in wastewater [15]. Gormely et al. reported that the sanitary plumbing system was one potential spread route for SARS-CoV-1 if infected patients were found in the community [16], and similarly SARS-CoV-2 virus can be transmitted by aerosol or water droplets. Additionally, SARS-CoV-2 biomarkers can be detected in the sewer system, because the virus can be isolated from the infected patients' feces and urine [17]. According to the results of various studies, which investigated fecal samples from infected patients, the positive rate of SARS-CoV-2 in patients' feces varied from 15 to 83% [18]. Especially, one study highlighted that the virus in feces persisted positively even after the nasopharyngeal testing had become negative [19]. Consequently, the analysis of wastewater in communities is a potential method to track infected people, and the epidemiology of the communities can be monitored via sewage pipe networks.

The concept of wastewater-based epidemiology is to give comprehensive health information on communities, and the schematic diagram of WBE is presented in Fig. 1 [20]. Initially, WBE was used to estimate drug abuse in the community. By analyzing certain target drug residues in sewage, it is possible to estimate and monitor the trends of illegal drug consumption [21]. Other biological indicators in wastewater, such as pharmaceuticals, and personal care products, markers of population size, and industrial chemicals, can also be quantitively and qualitatively determined to obtain epidemiologic data for addressing public health issues [22]. Similarly, viruses are another important biomarker for wastewater -based epidemiology. Since viruses cannot grow outside the host cells, the level of viruses in wastewater is directly proportional to the concentration of viruses in the excrement of the corresponding population [23]. Through the analysis of sewage, the changes in virus concentration and diversity can be obtained, which are useful information for assessing the temporal and spatial patterns of virus infection in the community. Significantly, adopting this method in the early stage of the viral outbreak can determine where the virus starts to develop and spread [1]. Correspondingly, early warning and intervention can be taken based on these findings. Considering all these merits of WBE, Bivins et al. call for global collaboration to improve detection approaches for COVID-19 in wastewater [24].

During the COVID-19 pandemic, there are several techniques for sewage analysis, and PCR-based methods have been the prime techniques in the application. Many countries reported the presence of SARS-CoV-2 RNA in sewage by using RT-PCR assays because as mentioned before, this diagnostic method provides high specificity and sensitivity. The Netherlands tested three nucleocapsid genes (N1–N3) and one the envelope gene (E) of SARS-CoV-2 in the sewage samples from six cities and airports [25]. The results showed that the target RNA was not detected before the first confirmed infection case in February. Nevertheless, due to the prevalence of COVID-19 in March, the target nucleic acid signal increased significantly. A study conducted in Massachusetts using RT-PCR also showed that the observed viral titers were higher than the expectation based on the confirmed clinic cases in March [26]. One explanation for this result may be that the clinic testing is limited in

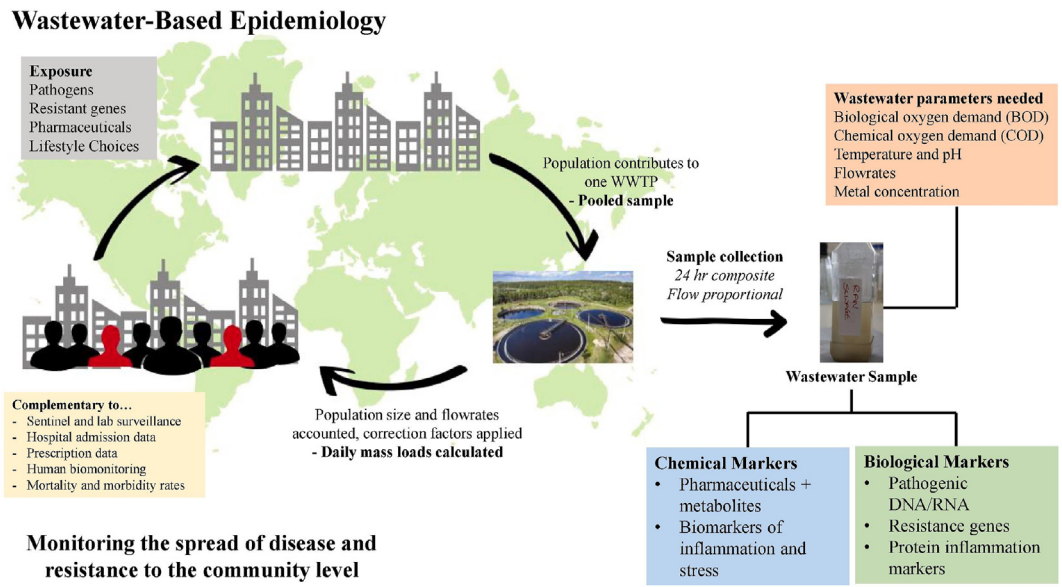

Fig. 1. The schematic diagram of WBE [20].

diagnosing asymptomatic patients. Therefore, using RT-PCR for viral detection in sewage can be regarded as an early warning tool for the presence and prevalence of COVID-19 infection and a supplementary method for clinical testing.

Although PCR-based methods are mainstream ones for sewage analysis, some disadvantages of PCR assay earlier mentioned limit its global application. When it comes to WBE, the ideal wastewater analysis method is to perform rapid virus detection at the wastewater collection outlets so that the transmission of the virus can be monitored in real time and early warnings can be implemented in time. Since PCR-based methods are time-consuming and need sophisticated equipment and centralized laboratories, it is necessary to develop other methods that have high specificity and sensitivity, but also have the advantages of low cost, rapidness, accuracy and simplicity [1]. One promising method that can be applied for WBE is a paper-based microfluidic device. This portable diagnostic device offers the potential for a medical diagnosis that can be used in resource-limited areas and urgent situations.

4. Paper-based devices

Paper has been used as a diagnostic tool for a long time, and the most common examples are test papers, such as pH test strips and urine test strips. The urine test strips are designed to detect some metabolic products in urine, which are common pathological indicators (e.g., protein, glucose, and salt) [27]. Due to the different concentrations of metabolic products, various color changes happened on the paper, and this can be read by comparing to the standard test strips.

With the improvement of rapid analysis methods, lateral flow assay (LFA) was introduced into paper-based diagnostic procedures and updated paper-based devices. One typical LFA device consists of four parts, involving sample pad, conjugate pad, nitrocellulose membrane and absorbent pad [28]. There are two types of LFA assay frequently used and they are competitive assay and sandwich assay. The former one is used to test low molecular weight or single epitope analytes, while the sandwich format is more suitable for analytes presenting several epitopes. When using competitive assay, if there is no analyte in the sample, unbound antibodies will bind to immobilized molecules on a sheet of the plastic backing, causing visual signal. On the contrary, a visual change can be observed on sandwich assay paper when the analyte is present in the sample. LFA, as a paper-based assay, gain popularity in the detection of drugs, toxins, pesticides, bacteria and viruses due to its simplicity and rapidity.

Currently, the latest paper-based devices refer to microfluidic devices that take advantage of paper as a substrate for various bioassays, and paper-based microfluidic devices are powerful tools for pathogen analysis and determination of infection transmission. The reason for choosing paper as the substrate is that it is affordable, light, easily accessible and disposable [15]. To fabricate paper-based microfluidic devices for different detection aims, paper made up with various material are picked up, such as cotton cellulose and nitrocellulose [27]. Also, there are several production approaches for paper-based microfluidic devices, including photolithography, polydimethylsiloxane (PDMS) plotting, inkjet printing, wax printing, wax screen printing, wax dipping, and plasma treatment.

When the biomolecules to be tested are nucleic acids, the frequently used approach is wax printing. The wax printer can print this analytical paper with wax channels that can restrict and guide samples and reagents, and the entire detection process of genetic materials, from extraction, enrichment, purification, elution, amplification, to visual detection are combined on this paper [15]. It is not required to have external power or pump supply for conducting paper-based testing, instead only a few simple folds of the device are necessary to complete the detection. Due to the simple operation and ease of fabrication, paper-based microfluidic devices can potentially be an alternative of PCR for rapid and accurate diagnosis of infectious disease with a fast turnaround time, when there is a lack of skilled professions and high-cost equipment.

Efforts have been made to improve paper-based microfluidics and studies showed that this technique meets prime demands of field-based diagnostics and can be used for infectious disease prevention, control and precise diagnosis. Kaarj et al. developed a platform utilizing RT-LAMP, a more straightforward amplification method requiring a constant temperature, to assay Zika virus (ZIKV), and the whole process was completed on a wax printed paper microfluidic chip as shown in Fig. 2 [29].

When the sample (urine or blood plasma) was added to the device, the paper fibers had a capability to filter large size molecules as a pretreatment. In addition, the viral RNA with strong negative charges spontaneously moved to the end of the channel, while proteins and other cell fragments were remained because of the strong negative polarity of cellulose fibers in paper. The target nucleic acids were amplificated on a simple hot plate, and the amplification results can be obtained by observing the intensity of the added pH indicator dye. The LAMP-based paper devices have also been used to diagnose rotavirus A, the most

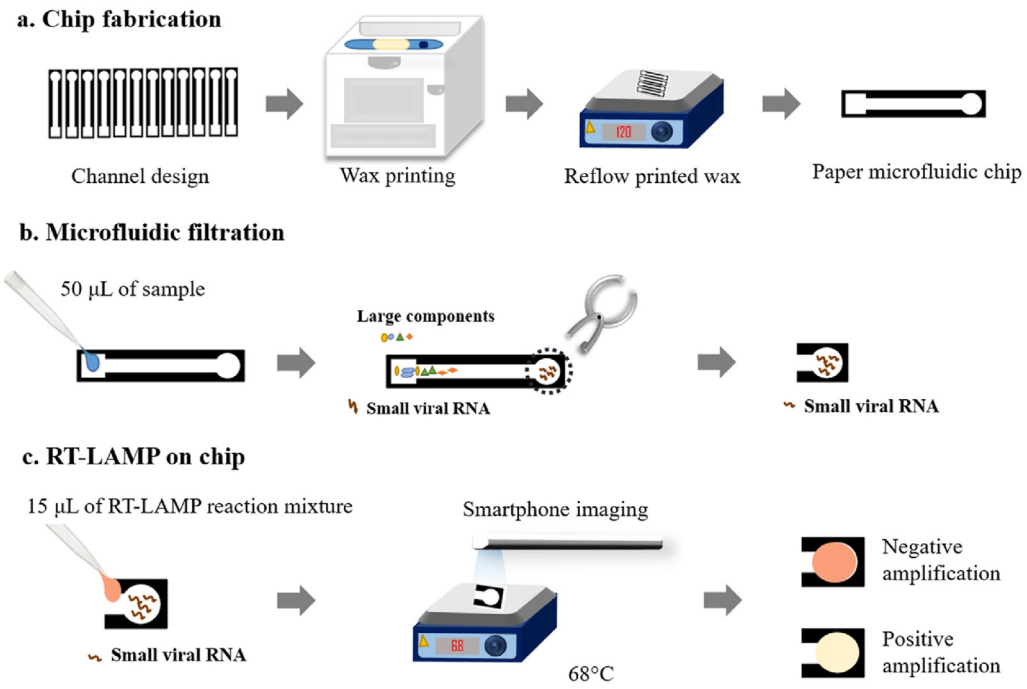

Fig. 2. The schematic diagram of paper microfluidic RT-LAMP assay for ZIKV [29].

common pathogen that contributes to gastroenteritis in children [30]. The nucleic acid extraction and thermostatic RNA amplification were completed on a simple paper disc, and the entire process only took about half an hour. The positive amplification result of rotavirus A can be read immediately with the naked eye, that is, the rose-red on the glass fiber paper.

The previously studies were to complete the detection of pathogens using paper-based microfluidic devices in the laboratory. In addition, one study practically used this point-of-care diagnostic tool in rural Uganda to quickly diagnose malaria for residents [31]. The testing could give results in approximately 50 min by analyzing multiplexed RNA sequences of pathogens, which greatly shortened the diagnosis time compared with the high-cost PCR analysis. Besides, the sensitivity of this diagnostic origami device was higher than expected, 98% of people with malaria can be detected, while the detection rates of other field-based, benchmark techniques, including optical microscopy and industry-standard rapid immunodiagnostic tests, were 86% and 83% respectively. Like PCR assay, these field-based techniques required experienced professions to perform.

Paper-based microfluidic devices were demonstrated to be rapid, affordable, portable and easy to handle in field-based diagnosis, and were feasible for use in resource-poor areas. Another advantage of the paper analytical tool is that it is thin and lightweight, thus this device tends to be easy to stack, transport and store [15]. Additionally, after using paper-based microfluidic device to complete the diagnosis, it can be disposed of by incineration, which possibly reduces the risk of causing contamination. As a result, this novel analytical device with various merits has excellent potential to be applied to respond to the COVID-19 pandemic, thereby enabling rapid and on-site virus detection for immediate diagnosis and wastewater-based epidemiology investigation.

5. Paper-based device for diagnostics and testing wastewater for early warning of COVID-19

5.1. Rapid diagnosis for COVID-19

One characteristic of COVID-19 is spreading quickly around the world, and a critical solution to this public health issue is to offer rapid and accurate screening for infected patients whether they have symptoms or not. Based on a large-scale screening of infection cases carried out early in the epidemic, confirmed cases can be treated in time, reducing severe cases, easing the burden on the medical system and managing the epidemic. Since paper-based microfluidic devices have been developed to diagnose infectious diseases before, it holds a great potential to meet the demand for rapid diagnosis for COVID-19 as well.

The first type of rapid diagnosis is a nucleic acid-based assay, and it is possible to design an analysis tool that can identify nucleic acids in a short time by applying various isothermal amplification techniques to paper-based devices.

Some paper-based devices have been focused on LAMP detection of nucleic acids in infectious diseases, which could be adopted to detect COVID-19 nucleic acids. For instance, Xu et al. presented a paper-origami device for multiplex detection of *Plasmodium falciparum*, *Plasmodium vivax* and *Plasmodium* pan [32]. DNA extraction, LAMP, and fluorescence detection was integrated for target detection from whole blood. Origami-paper based LAMP assay can identify between *Plasmodium falciparum* and *Plasmodium vivax* infections, which can guide appropriate treatments for corresponding infections. A paper microfluidic device has been developed for multiplex detection of three bovine infectious reproductive diseases in semen samples and tested in a rural India farm [33]. Pathogen DNA was extracted from bovine herpes virus-1 (BoHV-1), *Brucella* and *Leptospira*, then amplified by LAMP and detected fluorescently (Fig. 3). The detection limits for three pathogens were as low as 50 *Leptospira* organisms, 50 CFU *Brucella*, and 1 TCID50 BoHV-1, which make paper microfluidic devices suitable for on-site diagnosis of infectious diseases. Choi et al. developed an integrated paper-based biosensor for nucleic acid testing [34]. The FTA card and glass fiber were integrated into a lateral flow strip for sample addition, washing, paper-based LAMP, visual detection or smartphone quantification. A handheld battery-powered heating device was used for on-site LAMP tests. Detection limits of 10–1000 CFU mL^{-1} were measured for *Escherichia coli* in spiked drinking water, milk, blood and spinach. This integrated paper-based biosensor is suitable for transformation into a device for rapid on-site RT-LAMP detection of COVID-19 nucleic acids.

Recently, rapid progress has been made in RT-LAMP enabled isothermal amplification methods for COVID-19 diagnosis [35]. The

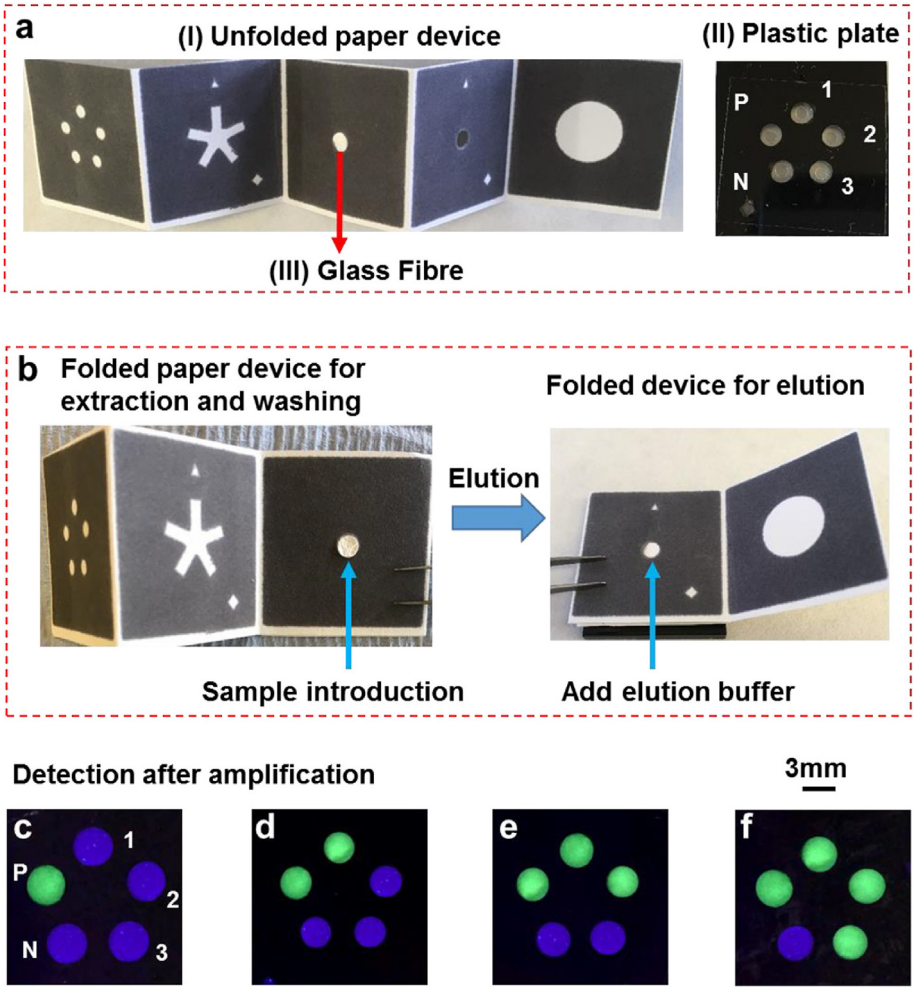

Fig. 3. The schematic diagram of the paper device for the detection of BoHV-1, Brucella and Leptospira [33].

Abbott recently launched the ID NOW COVID-19 assay for clinical use that can detect the RNA-dependent RNA polymerase (RdRp) gene from nasopharyngeal and throat swabs through isothermal amplification, and get a LOD of 125 genome equivalents mL^{-1} in less than 15 min [36]. Another team adopted the RT-LAMP and the clustered regularly interspaced short palindromic repeats (CRISPR) technique to detect SARS-CoV-2 in respiratory swabs within 40 min, and the diagnosis result can be known using the LFA, which is a conventional type of paper-based device [37]. This work has expanded the possibility of CRISPR-based DETECTR technology for on-site COVID-19 diagnosis with the investigations on portable microfluidic cartridges and lyophilized reagents. Therefore, it is promising to use paper-based devices such as LFA to detect the LAMP reaction products, and the whole process can be completed on a paper device for a point-of-care coronavirus disease diagnosis. Notably, the emergence of the paper-based devices for rapid diagnosis could be a massive improvement for places where resources are limited and the public health system is overwhelmed.

In addition to detecting nucleic acids to screen infected patients, paper-based devices can also be used to detect antibodies to help infectious disease diagnosis. Singh et al. reported a colorimetric paper-based sensor to detect pan malaria and *Plasmodium falciparum* based on a dye-based chromogenic reaction [38]. The instrument-free technique could be implemented for quantitative and qualitative determination of malaria by visual readout or a camera integrated software. In another work, a deployable bioplasmonic paper-based device was fabricated to detect *anti*-ZIKV-NS1 Immunoglobulin G (IgG) and IgM using LSPR [39]. The device was validated for target detection in serum samples, indicating great potential for effective determination of *anti*-ZIKV-NS1 IgG and IgM in the complex physiological environment with multiple interfering substances. When it comes to COVID-19, a variety of lateral flow test strips have been employed for COVID-19 diagnosis, such as Cellex qSARS-CoV-2 IgG/IgM Rapid Test, Wondfo SARS-CoV-2 antibody test, Mammoth Biosciences SARS-CoV-2 DETECTR and SGTi-flex COVID-19 IgM/IgG [40]. For example, a lateral flow immunoassay was presented for combined detection of SARS-CoV-2 IgG and IgM in human blood in less than 15 min during various infection stages, showing in Fig. 4 [41]. The detection device comprises of a sample pad, a conjugate pad, a nitrocellulose membrane, an absorbent pad and an adhesive card. The clinical efficacy uses was demonstrated by performing clinical tests on different venous and fingerstick blood samples with a sensitivity of 88.66% and a specificity of 90.63%.

Kasetsirikul et al. designed a colorimetric paper-based assay to detect SARS-CoV-2 humanized antibody, which is a cheap and accessible serological assay [42]. This development combined a common serological assay method, ELISA, with the paper-based devices, by coating the recombinant SARS-CoV-2 nucleocapsid antigen on the paper analysis tool. The target antibodies from human serum could be captured by the recombinant antigen on the device, forming an immunological complex. This complex formation could be read out through a colorimetric reaction, 3,3′,5,5′-tetramethylbenzidine substrate with horseradish peroxidase (TMB/HRP), and the entire process took 30 min, which was much shorter than a conventional ELISA assay (usually one to 2 h).

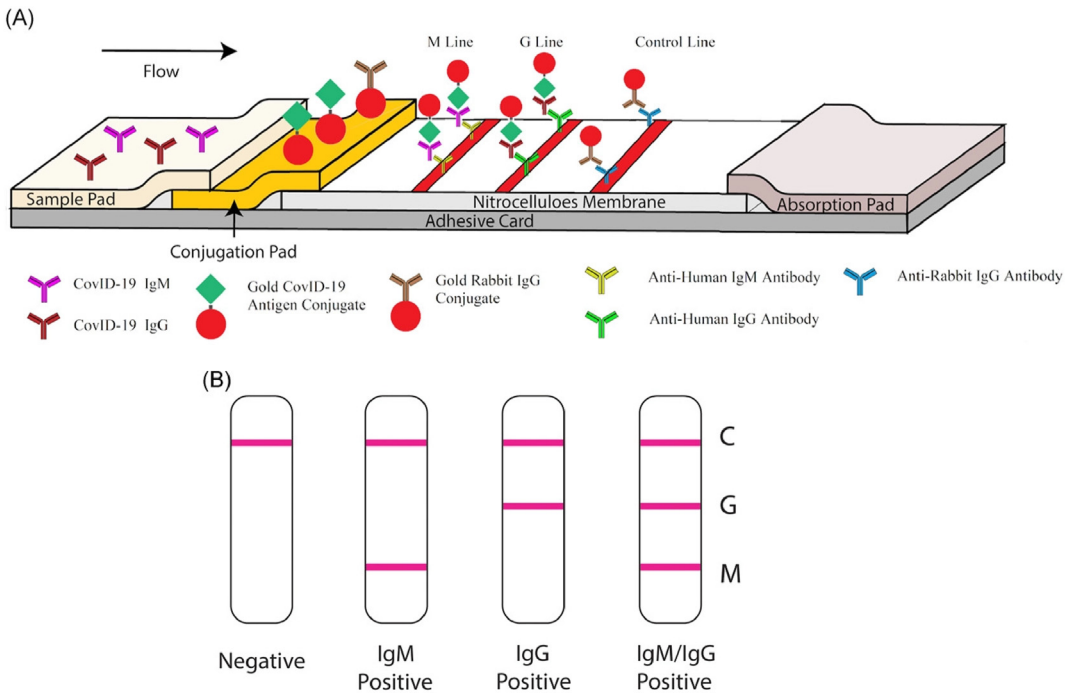

Fig. 4. The schematic illustration of SARS-CoV-2 IgG and IgM test [41]. (A) The detection device. (B) Different test results.

5.2. Analysis of SARS-CoV-2 for early warning of outbreak

The sewage analysis is a cost-effective and highly efficient method to determine the infectious cases, monitor the virus transmission and manage the pandemic. As mentioned before, PCR is still the main method used to detect SARS-CoV-2 in wastewater [44]. However, this kind of detection method that requires sophisticated instruments, complicated operation and long-time analysis is not suitable for real-time detection on the wastewater site. As an alternative approach, the paper-based device is suggested for on-site inspection of wastewater. Despite the fact that various kinds of substances contained in wastewater, it is believed that the paper-based device is capable of detecting pathogens from sewage.

Yang et al. reported a "sample-to-answer" tool to analyze the wastewater in communities, tracking biomolecular agents and genetic information [43]. The platform still took advantage of LAMP as a method of nucleic acid amplification. After removing solid impurities in the original wastewater and extracting and enriching DNA, it performed quantitative monitoring of the target genetic material. Finally, The LFA was used to present the visual assay result. The time to finish this assay at the sample collection point was 45 min, and no expensive equipment or experienced operators were required.

Similar paper devices may also become sewage analysis tools for the COVID-19 outbreak. In view of the high selectivity, high sensitivity, rapid response time and cost-effectiveness of this technique, it is believed that paper-based devices have the capability to be applied to the on-site detection of wastewater, providing real-time and continuous information. Based on this information, the local epidemiology of SARS-CoV-2 can be analyzed to better manage the infection situation in the community, such as early warning of epidemics or isolation of infected people.

5.3. Conclusion and future outlooks

In summary, during the COVID-19 outbreak, isolating the infected people through effective diagnostic methods is a universal method to slow the spread of the disease, and the main method used today is RT-PCR. Compared with RT-PCR, paper-based device is an analytical tool that can provide rapid and accurate biomolecular detection. More importantly, this detection method does not require laboratory-grade instruments and professional operators to complete it. The development of this cheap and rapid diagnosis method has brought hope to control the epidemic in some resource-limited regions and developing countries.

Besides, wastewater-based epidemiology is a new and promising mornitoring mechanism for managing epidemics at the community and population level, because the analysis of sewage can obtain the infection situation inside the area and facilitate early warning of infectious diseases, so as to carry out effective prevention and intervention. In order to continuously detect viral RNA in sewage in real time, an on-site detection method is needed. The paper-based device, such a portable analytical tool, can meet the requirements for wastewater-based epidemiology, and detect biomolecules in sewage containing complex matrices. Based on the analysis results, it is possible to give an early warning of infectious disease, identify SARS-CoV-2 carriers, determine the infection status in the community, and finally control the epidemic through timely measure and intervention.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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